[12013/58002]

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BOARD OF PATENT APPEALS AND INTERFERENCES

In re Application of:	Takafumi UENO et al.	Examiner: Christopher H. Yaen
For:	THERAPEUTIC ANGIOGENESIS BY BONE MARROW-DERIVED CELL TRANSPLANTATION IN MYOCARDIAL ISCHEMIC TISSUE AND SKELETAL MUSCLE ISCHEMIC TISSUE	Art Unit: 1642
Filed:	July 26, 2001	
Serial No.:	09/915,853	

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Elizabeth M. Wieckowski
Elizabeth M. Wieckowski (Reg. No. 42,226)

APPEAL BRIEF TRANSMITTAL

SIR:

Transmitted herewith for filing in the above-identified patent application, please find an Appeal Brief pursuant to 37 C.F.R. § 1.192(a), in triplicate.

Please charge the Appeal Brief fee of $330.00, and any other fees that may be required in connection with this communication to the deposit account of **Kenyon & Kenyon**, deposit account number **11-0600**.

Appellants hereby request a one-month extension of time, from August 4, 2004 to September 4, 2004, for submitting the Appeal Brief. Under 37 C.F.R. §1.7 when the day for taking any action or paying any fee in the U.S.P.T.O. falls on a Saturday, Sunday or on a Federal holiday within the District of Columbia, the action may be taken on the next succeeding business day which is not a Saturday, Sunday or on a Federal holiday. September 4, 2004 is a Saturday and Monday, September 6, 2004 is a Federal holiday. Accordingly, Appellants are timely submitting this Appeal Brief on Tuesday, September 7, 2004.

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Respectfully submitted,

Dated: September 7, 2004

By: Elizabeth M. Wieckowski

Elizabeth M. Wieckowski
Registration No. 42,226
KENYON & KENYON
One Broadway
New York, NY 10004
(212) 425-7200 (phone)
(212) 425-5288 (fax)
CUSTOMER NO. 26646

Docket No. 12013/58002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
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APPLICANTS	:	T. UENO et al.
SERIAL NO.	:	09/915,853
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FOR	:	THERAPEUTIC ANGIOGENESIS BY BONE MARROW-DERIVED CELL TRANSPLANTATION IN MYOCARDIAL ISCHEMIC TISSUE AND SKELETAL MUSCLE ISCHEMIC TISSUE
EXAMINER	:	C. H. Yaen
ART UNIT	:	1642

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Signature: Chandra Leonard

APPEAL BRIEF

Appellants respectfully submit this Appeal Brief in support of their appeal from the April 6, 2004 final rejection in this application.

Real Party in Interest

The real party in interest is the assignee of record, SCIMED Life Systems, Inc.

Related Appeals and Interferences

No other appeals or interferences are known to the Appellants, or to the Assignee or the Assignee's legal representatives involved in the prosecution of this application, which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

Status of Claims

The Appellants appeal the rejection of claims 2, 5, 7, 9-11, 13-15, 18, 20, 22, 24, 28-29, 31, 33, 38, 40-41 and 43.

During prosecution, claims 1, 3-4, 6, 8, 12, 16-17, 19, 21, 23, 25-27, 30, 32, 34-37, 39, 42 and 44-73 were canceled without prejudice and are not being appealed.

Status of Amendments

The last entered claim amendments were filed on December 9, 2003, in the paper entitled "Amendment in Response to September 9, 2003 Office Action."

The Appendix to this brief contains pending claims 2, 5, 7, 9-11, 13-15, 18, 20, 22, 24, 28-29, 31, 33, 38, 40-41 and 43, as they stood after the entry of the December 9, 2003 paper, and as currently appealed.

Summary of the Invention

As discussed in the background of the application, neovascular formation in adults has been thought to result exclusively from preexisting endothelial cells (ECs) by a process known as angiogenesis. See specification, page 1, lines 17-19. In contrast, the formation of new blood vessels from endothelial progenitor cells (EPCs) during embryogenesis, a process known as vasculogenesis, begins by formation of blood islands which comprise EPCs and hematopoietic stem cells (HSCs). See specification, page 1, lines 20-25. Circulating EPCs have been shown in adult peripheral blood and human umbilical cord blood and have been shown to participate in postnatal neovascularization after mobilization from bone marrow (BM). See specification, page 2, lines 1-5.

The present invention investigated whether functional EPCs may develop from BM mononuclear cells (BM-MNCs) in adult animals and whether transplantation of autologous BM-MNCs augments neovascularization in response to tissue ischemia in a pig model of chronic myocardial ischemia. See specification page 2, lines 21-25.

Appellant's invention relates to methods of forming new blood vessels in cardiac tissue by transplanting locally into the cardiac muscle tissue autologous BM mononuclear cells (BM-MNCs), which have been isolated from the subject's bone marrow. The administration of isolated autologous BM-MNCs which results in new blood vessel formation is used in the present invention in methods to increase blood flow to cardiac muscle, to increase angiogenesis in diseased cardiac muscle tissue, and to treat diseased cardiac muscle

tissue and heart failure.

Issues

Are claims 2, 5, 7, 9-11, 13-15, 18, 20, 22, 24, 28-29, 31, 33, 38, 40-41 and 43, which stand rejected under 35 U.S.C. §103, patentable over Toshiro Kobayashi et al., *Enhancement of Angiogenesis by the Implantation of Self Bone Marrow Cells in a Rat Ischemic Heart Model*, 89 J. SURGICAL RES. 189 (2000). (referred to herein as "Kobayashi").

Grouping of Claims

The entire group of the rejected claims for which this appeal is taken stand or fall together.

Argument

Claims 2, 5, 7, 9-11, 13-15, 18, 20, 22, 24, 28-29, 31, 33, 38, 40-41 and 43 stand rejected under 35 U.S.C. §103 as being obvious over Kobayashi. Appellants respectfully submit that a *prima facie* case of obviousness has not been established by the disclosure of Kobayashi.

In order to establish a *prima facie* case of obviousness, three criteria must be met. First there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference must teach all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, not in applicant's disclosure. *See In re Vaeck*, 947 F.2d 488 (Fed. Cir. 1991).

A. Kobayashi Fails to Suggest or Motivate Modification of Its Disclosure to Isolate Autologous Mononuclear Cells From Bone Marrow as Recited in Independent Claims 9, 22, 31, 38 and 43.

Kobayashi acknowledges that bone marrow contains many kinds of immature cells which could differentiate into hematopoietic cells and endothelial progenitor cells. See Kobayashi at 189. Kobayashi refers to Takayuki Asahara et al., *Isolation of Putative*

Progenitor Endothelial Cells for Angiogenesis, 275 SCIENCE 964 (1997) (referred to herein as "Asahara") in support of this acknowledgment:

> **A subset of CD-34 positive cells[1] derived from bone-marrow have the capacity to differentiate into endothelial cells** *in vitro* and be induced into the inner surface of small vessels *in vivo*. [5] *See* Kobayashi at 189. (Appellants' emphasis)

Appellants respectfully note that ***not all mononuclear cells are CD34-positive.***[2] (Appellants' emphasis) In fact, an extremely small percentage --1% to 4%-- of normal bone marrow mononuclear cells are CD34-positive cells. *See* Yasuhiro Deguchi & John H. Kehri, *Selective Expression of Two Homeobox Genes in CD34-Positive Cells from Human Bone Marrow*, 78 BLOOD 323 (1991), attached as Exhibit 1. Appellants' invention is not directed to isolating the minuscule subset of mononuclear cells which are CD34+ cells from the bone marrow. Kobayashi's reference to Asahara's teaching of CD34-positive mononuclear cells would not guide one of skill in the art to isolate the mononuclear cells from bone marrow cells, as claimed, since, as discussed below. As stated in the Abstract, page 189, col. 2 and at page 194, col. 2, last sentence, Kobayashi proposes a simple method that does not require further isolation of any subset of cells found within the bone marrow, other than the initial removal of red blood cells. As such, Kobayashi does not suggest any separation of cell types generally or specifically from the isolated bone marrow which is transplanted.

Even if Kobayashi suggested the isolation of only CD34+ mononuclear cells, the suggestion would lead one of ordinary skill in the art away from appellants' invention, since appellants' method isolates the entire fraction of mononuclear cells, the majority of which are CD34-, and does not further separate CD-34+ hematopoietic stem cell fraction from the mononuclear cells.

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[1]Asahara isolates CD34-positive mononuclear cells from human peripheral blood by means of magnetic beads coated with antibody to CD34. *See* Asahara, *supra*, at 964, Col. 1, ¶4 - col. 2.

[2]Human hematopoietic stem and progenitor cells are identified with a characteristic surface membrane glycoprotein, which has been defined as the CD34 antigen. *See* Yasuhiro Deguchi & John H. Kehri, *Selective Expression of Two Homeobox Genes in CD34-Positive Cells from Human Bone Marrow*, 78 BLOOD 323 (1991). The CD34-positive cells found in normal bone marrow represents 1% to 4% of the total number of bone marrow mononuclear cells. *See Id.*

Further support for appellants' position that Kobayashi does not teach or suggest the isolation of any specific subset of cells contained within the bone marrow is found in Kobayashi's setting forth of the two-fold reason for selecting bone marrow cells --not the mononuclear fraction or the CD34+ subfraction thereof-- to induce angiogenesis:

> In the present study, we selected bone marrow cells as the material for inducing angiogenesis because they contain endothelial progenitor cells that can participate in vascular formation in severe ischemic lesions (citing Asahara), and they are able to secrete a variety of growth factors such as VEGF and bFGF. *See* Kobayashi at 193. (Appellants' emphasis)

Thus, Kobayashi does not teach or suggest that ***any particular cell type be removed*** from the bone marrow's mixture of various cells –since both endothelial progenitor cells and cells which secrete various growth factor are deemed necessary-- for implantation into an ischemic heart model to induce angiogenesis. Neither is there any teaching or suggestion that ***mononuclear cells***, specifically, be removed from the bone marrow for such transplantation.

Kobayashi's discussion of factors which induce angiogenesis states:

> ... the protein expression of inflammatory cytokine, such as IL-1β and CINC, which have been proven to have angiogenic potency *in vitro* and *in vivo*, was significantly increases in the BMI group compared with the other groups [19-22]. **The main potential source for these inflammatory cytokines are monocytes, macrophages, and granulocytes, all of which the bone marrow contains**. *See* Kobayashi at 194. (Appellants' emphasis)

Kobayashi then suggests that the angiogenesis observed by the bone marrow implantation in his study depends on those inflammatory cytokines: "It is suggested that angiogenesis induced by the BMI treatment in this model is related to inflammatory cytokines, but not VEGF or bFGF." *See* Kobayashi at 194. This statement suggests the importance of including monocytes, macrophages, and granulocytes, since these cells produce the inflammatory cytokines upon which angiogenesis depends. At most, these statements by Kobayashi would lead one of ordinary skill in the art to attempt to isolate all of the aforementioned monocyte,

macrophage, and granulocyte cell types. However, Kobayashi does not teach or suggest that any one of these cell types be isolated from the bone marrow cells containing all of them for induction of angiogenesis.

As is well known, mononuclear cells include lymphocytes and monocytes, not granulocytes; whereas, granulocytes, which include eosinophils, neutrophils and basophils, are polymorphonuclear cells, i.e. have a complexly lobed nucleus. *See, e.g.,* Janis Kuby, IMMUNOLOGY (3rd ed., W.H. Freeman) (1997) *available at* http://www.whfreeman.com/ immunology/CH03/kuby03a.htm, attached as Exhibit 2; THE DICTIONARY OF CELL AND MOLECULAR BIOLOGY - ONLINE!, *at* http http://www. mblab.gla.ac.uk /~julian/dict2.cgi?4169 and *at* http://www.mblab.gla.ac.uk/~julian/dict2.cgi?4885, attached as Exhibit 3; and Figure 3-2 from RICHARD I. WALKER ET AL., MEDICAL CONSEQUENCES OF NUCLEAR WARFARE (Richard I. Walker & T. Jan Cerveny eds., TMM Publications, Office of the Surgeon General;1989) *available at* http://afrri.usuhs.mil/www/ outreach/pdf/tmm/Chapter 3/figure3-2.pdf, attached as Exhibit 4. Accordingly, even if Kobayashi's teachings suggested the isolation of monocytes, macrophages, and granulocytes from the bone marrow, the suggestion would lead one of ordinary skill in the art away from the present invention, since by definition, mononuclear cells do not include granulocytes.

Therefore, contrary to the Examiner's assertion, one of skill would not have any motivation to ***isolate MNCs from bone marrow*** based on Kobayashi's teaching because only the administration of BM cells, not MNCs isolated therefrom, is taught by Kobayashi for induction of angiogenesis. Even if Kobayashi's disclosure suggested isolation of either CD34+ cells or the combination of monocytes, macrophages, and granulocytes, these suggestions would lead one of skill away from appellants' invention.

B. Kobayashi Does Not Provide a Reasonable Expectation that Isolated BM-MNCs Would Form New Blood Vessels.

One of skill in the art would not have a reasonable expectation that implantation of BM-MNCs would form new blood vessels and/or induce angiogenesis, since Kobayashi's results are obtained with BM cells from which only red blood cells have been removed without any further separation of MNCs therefrom.

Further, Kobayashi speculation, as discussed above, that "possibly, unknown growth factors might account for the angiogenesis induced by transplantation of bone marrow" would suggest to one of skill that the **entire** fraction of bone marrow cells is required for

administration so as to retain the unknown factors in order to successfully form new blood vessels and/or induce angiogenesis.

Moreover, Kobayashi's reference to the potential sources for inflammatory cytokines in the bone marrow, i.e. monocytes, macrophages, and granulocytes, would suggest that all of these cell types may be required for new blood vessel formation and/or induction of angiogenesis, rather than only mononuclear cells, as claimed.

Kobayashi's conclusion that "[f]urther investigations on BMI treatment are required to clarify the optimal populations of whole bone marrow cells that will have the most angiogenic potency and to determine whether BMI will induce stronger angiogenesis in an ischemic environment" would strongly suggest experimentation with whole bone marrow, rather than ***any one specific cell type or mononuclear cells, in particular***, for successful angiogenesis. *See* Kobayashi at 194.

Accordingly, Kobayashi provides no reasonable expectation of success that administration of MNCs isolated from the bone marrow would form new blood vessels and/or induce angiogenesis, as claimed.

C. Kobayashi Does Not Teach All the Claim Limitations

The present invention is directed to methods of forming new blood vessels in cardiac muscle tissue in a subject, methods of increasing blood flow to cardiac muscle tissue in a subject, methods of treating diseased cardiac muscle tissue in a subject, methods of increasing angiogenesis in diseased cardiac muscle tissue in a subject, and methods of treating heart failure in a mammal, wherein the subject is a human, which methods comprise: a) isolating autologous bone marrow-mononuclear cells from the human, ***wherein the autologous bone marrow-mononuclear cells are isolated from bone marrow***; and b) transplanting locally into the cardiac muscle tissue an effective amount of the autologous bone-marrow mononuclear cells, resulting in formation of new blood vessels in the cardiac muscle tissue. (Emphasis added) Therefore, the claims specifically recite BM-MNCs which have been isolated, *i.e.*, **removed from bone marrow cells** which contain various cell types, as well as other components. (Emphasis added)

As noted above, Kobayashi's Abstract and Discussion both conclude by specifically stating that "bone marrow implantation could be a novel and simple method to induce therapeutic angiogenesis". Accordingly, Kobayashi provides no teaching of isolation of any

particular cellular component, generally, or MNCs specifically, from the bone marrow. Rather, Kobayashi's disclosure supports the use of bone marrow, as a simple method that does not require further isolation of any subset of cells found within the bone marrow, other than the initial removal of red blood cells. Therefore, Kobayashi does not teach all of the limitations as claimed.

Accordingly, a *prima facie* case of obviousness has not been established by Kobayashi.

For the foregoing reasons, the Appellants respectfully submit that the rejection of the pending claims should be reversed.

Fee Authorization

The Commissioner is authorized to charge the fee for this appeal brief of $330.00 as set forth in 37 C.F.R. § 1.17(c), as well as any other applicable fee, to Deposit Account No. 11-0600.

Respectfully submitted,

KENYON & KENYON

Dated: September 7, 2004

Elizabeth M. Wieckowski
Reg. No. 42,226

One Broadway
New York, N.Y. 10004
212-425-7200
212-908-6140 (Direct)
212-425-5288 (Fax)

Appendix

2. (Previously amended) The method of claim 9, wherein the cardiac muscle tissue is ischemic cardiac muscle tissue.

5. (Previously amended) The method of claim 9, wherein the cardiac muscle tissue is damaged cardiac muscle tissue.

7. (Previously amended) The method of claim 5, wherein the damaged cardiac muscle tissue is an artificially created site.

9. (Previously amended) A method of forming new blood vessels in cardiac muscle tissue in a subject, wherein the subject is a human, which comprises:

a) isolating autologous bone marrow-mononuclear cells from the human, wherein the autologous bone marrow-mononuclear cells are isolated from bone marrow; and

b) transplanting locally into the cardiac muscle tissue an effective amount of the autologous bone-marrow mononuclear cells, resulting in formation of new blood vessels in the cardiac muscle tissue.

10. (Previously amended) The method of claim 9, wherein the new blood vessels comprise capillaries.

11. (Previously amended) The method of claim 9, wherein the new blood vessels comprise collateral vessels.

13. (Previously amended) The method of claim 22, wherein the new blood vessels comprise capillaries.

14. (Previously amended) The method of claim 22, wherein the new blood vessels comprise collateral blood vessels.

15. (Previously amended) The method of claim 22, wherein the cardiac muscle tissue is

ischemic cardiac muscle tissue.

18. (Previously amended) The method of claim 22, wherein the cardiac muscle tissue is damaged cardiac muscle tissue.

20. (Previously amended) The method of claim 18, wherein the damaged cardiac muscle tissue is an artificially created site.

22. (Previously amended) A method of increasing blood flow to cardiac muscle tissue in a subject, wherein the subject is a human, which comprises:

a) isolating autologous bone-marrow mononuclear cells from the human, wherein the autologous bone marrow-mononuclear cells are isolated from bone marrow; and

b) transplanting locally into the cardiac muscle tissue an effective amount of the autologous bone-marrow mononuclear cells so as to result in formation of new blood vessels in the cardiac muscle tissue, thereby increasing the blood flow to the cardiac muscle tissue in the human.

24. (Previously amended) The method of claim 31, wherein the diseased cardiac muscle tissue is ischemic cardiac muscle tissue.

28. (Previously amended) The method of claim 31, wherein the new blood vessels comprise capillaries.

29. (Previously amended) The method of claim 31, wherein the new blood vessels comprise collateral blood vessels.

31. (Previously amended) A method of treating diseased cardiac muscle tissue in a subject, wherein the subject is a human, which comprises:.

a) isolating autologous bone-marrow mononuclear cells from the human, wherein the autologous bone marrow-mononuclear cells are isolated from bone marrow; and

b) transplanting locally into the diseased cardiac muscle tissue an effective amount of the autologous bone-marrow mononuclear cells so as to result in formation of new blood vessels, thereby treating the diseased cardiac muscle tissue in the human.

33. (Previously amended) The method of claim 38, wherein the diseased cardiac muscle tissue is ischemic cardiac muscle tissue.

38. (Previously amended) A method of increasing angiogenesis in diseased cardiac muscle tissue in a subject, wherein the subject is a human, which comprises:

a) isolating autologous bone-marrow mononuclear cells from the human, wherein the autologous bone marrow-mononuclear cells are isolated from bone marrow; and

b) transplanting locally into the diseased cardiac muscle tissue an effective amount of the autologous bone-marrow mononuclear cells, thereby increasing angiogenesis in the diseased cardiac muscle tissue in the human.

40. (Previously amended) The method of claim 43, wherein the new blood vessels comprise capillaries.

41. (Previously amended) The method of claim 43, wherein the new blood vessels comprise collateral blood vessels.

43. (Previously amended) A method of treating heart failure in a mammal, wherein the subject is a human, which comprises:

a) isolating autologous bone-marrow mononuclear cells from the human, wherein the autologous bone marrow-mononuclear cells are isolated from bone marrow; and

b) transplanting locally into the heart an effective amount of the autologous bone-marrow mononuclear cells so as to result in formation of new blood vessels, thereby treating heart failure in the human.

Selective Expression of Two Homeobox Genes in CD34-Positive Cells From Human Bone Marrow

By Yasuhiro Deguchi and John H. Kehrl

Proteins coded by homeobox-containing genes are sequence-specific DNA-binding proteins that have been implicated in the control of gene expression both in developing as well as in adult tissues. Two recently characterized human homeobox genes, HB24 and HB9, were found to be highly expressed in bone marrow cells enriched for CD34-positive cells, present at low levels in unfractionated bone marrow cells, and essentially undetectable in bone marrow cells depleted of CD34 cells. Treatment of CD34-enriched cells with recombinant interleukin-3 (IL-3) and granulocyte macrophage-colony-stimulating factor for 24 hours increased expression of HB24 threefold and HB9 fourfold. Based on studies with actinomycin D, the HB24 and HB9 transcripts in human CD34-positive cells have short half-lives, estimated to be 30 to 45 minutes. Downregulation of HB24 and HB9 expression was found following the treatment of in vitro cultures of CD34-positive cells with IL-3. Thus, the differentiation of CD34-positive cells along a specific cell lineage likely requires downregulation of both HB24 and HB9.

HUMAN HEMATOPOIETIC stem and progenitor cells belong to a subset of undifferentiated bone marrow (BM) cells that have a unique surface membrane glycoprotein of 110 to 120 Kd. This glycoprotein is recognized by several monoclonal antibodies (MoAbs), My10, 12.8, BI.3C5, and ICH3, and has been defined as the CD34 antigen.[1-4] CD34-positive cells are found in the normal BM; represent 1% to 4% of the total number of BM mononuclear cells; and comprise virtually all the hematopoietic progenitor cells that are capable of forming granulocytic (colony-forming unit-granulocyte-macrophage [CFU-GM]), erythroid (burst-forming unit-erythroid [BFU-E]), megakaryocytic (CFU-Mk), multi-lineage (CFU-mix), and blast cell colonies (CFU-blast).[1-4] Transplantation of a population of BM cells enriched for CD34-positive cells into lethally irradiated baboons or cancer patients treated with ablative chemotherapy completely restores hematopoiesis.[5,6] While many of the cytokines and cytokine receptors important in differentiation of stem cells and progenitor cells to mature hematopoietic cells have been recently defined,[7] the molecular mechanisms and transcriptional factors important in this differentiation process are largely unknown. Recent studies have identified certain common features present in transcriptional factors known to regulate gene transcription in other cell types. For example, several different motifs (ie, helix-turn-helix, zinc fingers, and helix-loop-helix) used to bind specific DNA sequences have been identified.[8]

We have recently isolated cDNAs for two putative transcriptional factors, HB9 and HB24, from an activated human B-cell cDNA library that are expressed in activated but not resting lymphocytes, in approximately 5% of BM cells, and in certain developing tissues[9] (Y. Deguchi, manuscripts submitted for publication). Both these genes are members of a family of DNA-binding proteins with a characteristic 180-bp protein-coding sequence called a homeobox. Homeobox genes were originally identified as encoding for proteins important in Drosophila embryonic development and body segmentation.[10] The carboxy terminus of the homeodomain exhibits similarity to the helix-turn-helix motif of DNA-binding regulatory proteins of prokaryotes and yeast, and homeodomain-containing proteins have been shown to be sequence-specific DNA-binding proteins that regulate transcription.[11,12] Homeobox genes are broadly conserved evolutionarily and have been isolated from a variety of species, including mammals.[13,14] While the majority of mammalian homeodomain proteins are closely related (80% to 90%) to the major classes of Drosophila homeodomain proteins, HB9 and HB24 are moderately diverged and are only 38% to 58% identical.

Because the number of HB9- or HB24-positive cells found in unfractionated BM cells was similar to the number of CD34-positive cells known to be present in BM, the expression of HB9 and HB24 in purified CD34 cells was investigated by RNase protection assay and in situ hybridization. Furthermore, we examined the changes in HB9 and HB24 expression following the differentiation of purified CD34 cells in vitro. The significance of these results is discussed.

MATERIALS AND METHODS

Preparation of BM suspensions and purified CD34-positive cells. BM aspirates were obtained from the posterior iliac crest of normal volunteers after informed consent. The samples were immediately diluted 1:1 with Iscove's modified Dulbecco's medium (IMDM; GIBCO Laboratories, Grand Island, NY) containing 20 U/mL of sodium heparin. This mixture was passed through a 150-μm screen and layered over an equal volume of Ficoll-Paque (specific gravity, 1.077 g/cm^3; Pharmacia Fine Chemicals, Piscataway, NJ). Density centrifugation was performed at 500*g* for 25 minutes at 4°C and the interface layer of low density mononuclear cells was collected, washed, and resuspended in phosphate-buffered saline (PBS)-EDTA (PBS, pH 7.4 containing 5% fetal bovine serum [FBS], vol/vol; 0.01% EDTA, wt/vol; and 1.0 g/L D-glucose). The low density marrow cells were treated with anti-HPCA-1 (My10) murine monoclonal antibody (MoAb; Becton Dickinson, Mountain View, CA) for 30 minutes at 4°C, washed three times, and mixed

From the Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD.

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Address reprint requests to Yasuhiro Deguchi, MD, National Institutes of Health, Building 10, Room 11B-13, Bethesda, MD 20892.

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with sheep antimouse Ig-conjugated magnetic beads (Dynabeads; Dynal, Oslo, Norway) at a ratio of 1:20 (cell to bead).[15] The CD34-positive cells were separated by a cobalt-samarium magnet-equipped device (Dynabeads).[15] After separation, the cells were washed twice with PBS and used for preparation of cytoplasmic RNA. The purity of the magnetic bead sorted cells was determined by re-analyzing aliquots of the separated subsets on a Coulter Epics 753 (Coulter, Hialeah, FL). The CD34-enriched fraction contained greater than 92% My10-positive cells and the CD34-depleted fraction contained less than 0.8% My10-positive cells, respectively. The low-density BM cell fraction before the separation contained approximately 2.8% My10-positive cells.

RNase protection assay. Cytoplasmic RNA was isolated from BM cells using a miniprep method.[16] Briefly, cells were washed once with ice-cold tris-saline (25 mmol/L Tris pH 7.4, 130 mmol/L NaCl, 5 mmol/L KCl), spun at low speed in an Eppendorf centrifuge (5,000 rpm) at 4°C for 1 minute, resuspended in 400 μL of tris-saline, and 100 μL of NDD buffer (1% NP-40, 0.5% sodium deoxycholate, and 0.02% dextran sulfate in tris saline) was added to the suspended cells. The tubes were inverted 10 times and spun at low speed as above. The supernatant was transferred to a fresh tube and 500 μL of phenol/chloroform, 20 μL of 20% sodium dodecyl sulfate (SDS), and 15 μL of 5 mol/L NaCl were added. After centrifugation at high speed (12,000 rpm), the upper phase was collected, the phenol/chloroform extraction repeated until the interface was clear, and a last extraction with chloroform alone was performed to remove any residual phenol. The cytoplasmic RNA was ethanol precipitated and resuspended in diethylpyrocarbonate-treated water and frozen until use. The RNase protection assays were performed using standard methodology.[17,18] An HB24 *Eco*RI-*Sma* I fragment (351 bp) and HB9 *Eco*RI-*Xho* I fragment (295 bp) were each subcloned into pBluescript (Stratagene, La Jolla, CA). An actin *Bam*HI-*Hin*d III fragment (145 bp) from the 3′ noncoding region of a γ-actin cDNA was also subcloned into pBluescript.[19] ^{32}P-labeled HB24, HB9, and control actin RNA transcripts were made in the sense and anti-sense direction and hybridized to 10 μg of total cytoplasmic RNA. Cytoplasmic RNA transcripts protected from RNase digestion were size-fractionated on polyacrylamide gels and visualized by autoradiography. No fragments were protected with the sense probes.

In vitro culture of CD34 cells. The CD34-positive cells were cultured in IMDM (GIBCO) supplemented with 5% bovine calf serum (Flow Laboratories, Inc, McLean, VA) and recombinant interleukin-3 (IL-3) (200 U/mL) in 24-well plates (Costar, Cambridge, MA) for 3 weeks in a CO_2-incubator at 37°C. Fresh media was added twice a week. Cytospin slides of both untreated CD34-positive cells and differentiated cells were prepared for in situ hybridization.

In situ hybridization to analyze HB24 and HB9 gene expression. The HB24 and HB9 antisense RNA probe (^{35}S-labeled transcripts of HB24 *Eco*RI-*Sma* I 462 bp with T3 promoter and HB9 *Eco*RI-*Xho* I 406 bp with T7 promoter) detecting the sense HB24 and HB9 RNA or the HB24 and HB9 RNA sense probe (same fragment with T7 for HB24 and T3 promoter for HB9 were made by standard methods).[20] Slides were prepared, prehybridized, hybridized, and washed as previously described.[21] After 4 days of exposure to a 1:1 dilution of Kodak NTN-2 emulsion (Eastman Kodak, Rochester, NY), the slides were developed in Kodak D-19 and Rapid Fix, and stained with Hematoxylin-Eosin and Giemsa solution.

RESULTS

Analysis of HB24 and HB9 mRNA expression in CD34-positive BM cells. Examination of unfractionated BM cells for the expression of either HB24 or HB9 mRNA by in situ hybridization with anti-sense RNA probes previously showed hybridization in approximately 5% of the cells[9] (Y. Deguchi, manuscripts submitted for publication). Because homeobox gene mRNAs are often present at relatively low copy numbers and may be difficult to detect by standard Northern blots, a more sensitive RNase protection assay was used to study the expression of HB9 and HB24. Cytoplasmic RNA was extracted from unfractionated BM cells, highly enriched CD34-positive cells purified from BM, and unfractionated BM cells depleted of CD34-positive cells and analyzed by RNase protection assay using either HB24- or HB9-specific RNA probes. High levels of both the HB24 and the HB9 mRNA transcripts were detected in the highly enriched CD34-positive fraction, whereas low levels of each gene were evident in total BM cells, and very low levels of the transcripts were present in the fraction depleted of CD34-positive cells (Fig 1). A human actin

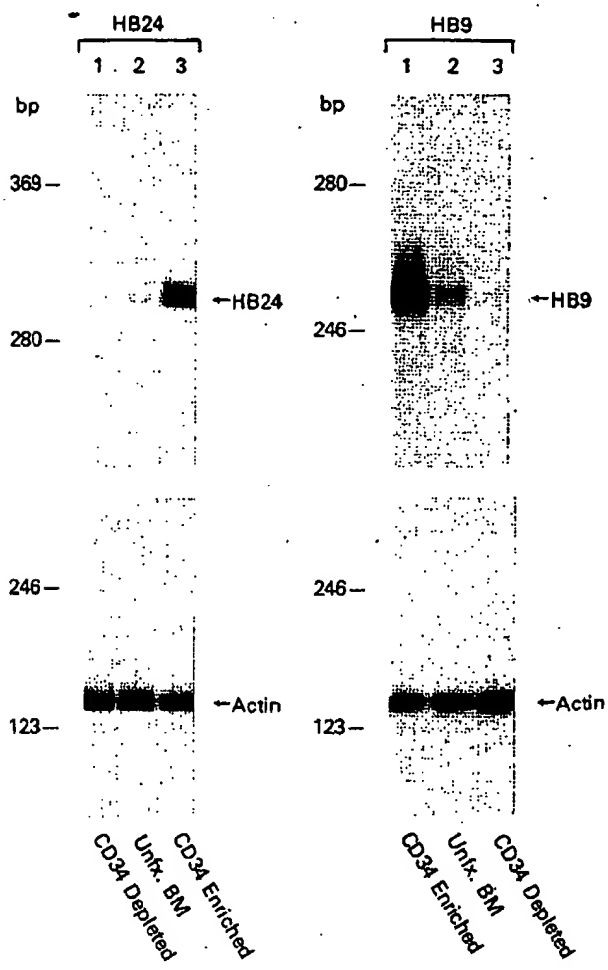

Fig 1. Expression of human homeobox genes HB24 and HB9 in CD34-positive human cells. RNase protection assays were performed to assess HB24 (top, left panel) and HB9 (top, right panel) expression (18 hours of exposure) and actin (lower panels) expression was used as a control (6 hours of exposure). Lane 1, CD34-depleted cells; lane 2, total unfractionated BM cells; and lane 3, CD34-positive–enriched mononuclear cell fraction. Lanes 1 and 3 are reversed for HB9. Ten micrograms of cytoplasmic RNA was analyzed per lane. The sizes of the protected fragments are indicated.

RNA probe was used as a control to ascertain that equal amounts of intact RNA were analyzed. The actin mRNA level in each RNA sample was similar (Fig 1). Quantification of expression levels by densitometric analysis and measurement of ^{32}P incorporation in excised bands with a beta counter showed that the levels of HB9 and HB24 transcripts in the CD34-positive–enriched fractions were approximately 40 times and 50 times higher than in the CD34-depleted fraction, respectively.

To determine the half-lives of HB24 and HB9 mRNA transcripts in CD34-positive cells, gene transcription was blocked by the addition of actinomycin D, and the levels of HB24 and HB9 mRNA transcripts were analyzed at various time intervals. The estimated half-lives of the HB24 and HB9 mRNAs in CD34-positive cells were approximately 45 minutes and 30 minutes, respectively (Fig 2). Inhibition of protein synthesis with cycloheximide for 1 hour did not significantly alter the HB24 and HB9 mRNA transcript levels in CD34-positive cells (Fig 2).

Further induction of HB24 and HB9 mRNA transcripts in IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF)-treated CD34-positive cells. Because the HB24 and HB9 mRNA transcripts are induced in lymphocytes activated with the appropriate stimuli such as phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) for T lymphocytes or *Staphylococcus aureus* cowan I (SAC) and PMA for B lymphocytes, the levels of HB24 and HB9 expression were examined following treatment of CD34-positive cells with recombinant IL-3 and GM-CSF. IL-3 and GM-CSF provide a potent proliferative and differentiative stimulus for hematopoietic progenitor cells.[22] Exposure of the CD34-enriched fraction to IL-3 and GM-CSF for 24 hours increased the levels of HB24 and HB9 mRNA transcripts approximately threefold (3.2-fold) and fourfold (4.3-fold), respectively, as determined by densitometric analysis and excision of the appropriate band for analysis on a beta counter (Fig 3). No morphologic changes suggestive of differentiation of CD34-positive–enriched BM cells occurred during the first 48 hours of these cultures as assessed by an inverted light microscope using standard criteria.[23,24]

Downregulation of HB24 and HB9 expression following the differentiation of CD34-positive cells in vitro. While the HB24 and HB9 mRNA transcripts are expressed highly in CD34-positive BM cells and can be further increased following a brief exposure to IL-3 and GM-CSF, the absence of HB24 and HB9 mRNA in CD34-depleted BM cells suggested that both of these genes are downregulated following differentiation. To examine whether the levels of HB24 and HB9 mRNA expression were altered in CD34 cells induced to differentiate in vitro, we examined the levels of HB24 and HB9 mRNA by in situ hybridization and RNase protection assay following in vitro treatment with recombinant IL-3 for 3 weeks. IL-3 is known to differentiate CD34-positive cells along the myeloid lineages.[22,25] CD34-positive cells were strongly HB24 and HB9 mRNA positive by in situ hybridization (Fig 4). No hybridization signal was found when either the HB24 or HB9 sense control probes were used (data not shown). When treated with IL-3 for 3 weeks, the CD34-positive cells differentiated as assessed by an inverted light microscope using standard criteria[23,24] and no longer had detectable levels of HB24 and HB9 mRNA by in situ hybridization (Fig 4). Downregulation of HB24 and HB9 mRNA expression was also confirmed following the differentiation of CD34-positive cells in vitro by RNase protection assay with RNAs from untreated and cultured cells (Fig 5).

DISCUSSION

Differentiation of hematopoietic progenitor cells along specific cell lineages must be accompanied by altered gene transcription of a variety of regulatory and tissue-specific genes to account for the appearance of various mature phenotypes.[25] We have identified two putative transcrip-

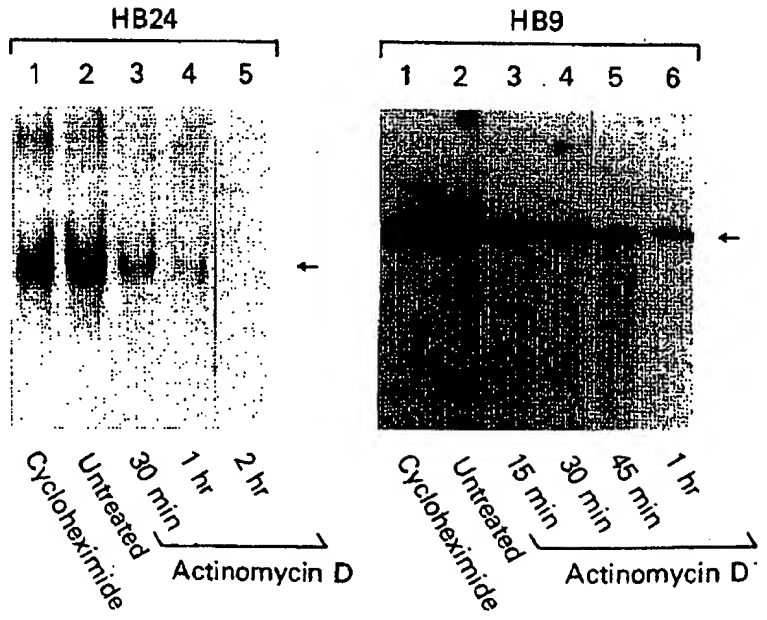

Fig 2. Effect of actinomycin D and cycloheximide on HB24 and HB9 expression in CD34-positive cells. RNase protection assays were performed to assess HB24 (left panel) and HB9 (right panel) expression. Lane 1, CD34-positive cells treated with cycloheximide (10 μg/mL) for 1 hour; lane 2, untreated CD34-positive human BM cells; lanes 3 through 5 for HB24– CD34-positive cells treated with actinomycin D (1 μg/mL) for 30 minutes, 1 hour, or 2 hours, respectively; and lanes 3 through 6 for HB9– CD34-positive cells treated with actinomycin D (1 μg/mL) for 15 minutes, 30 minutes, 45 minutes, and 1 hour, respectively. Ten micrograms of cytoplasmic RNA was analyzed in each lane. Half-lives of HB9 and HB24 were estimated by assuming an exponential rate of decay.

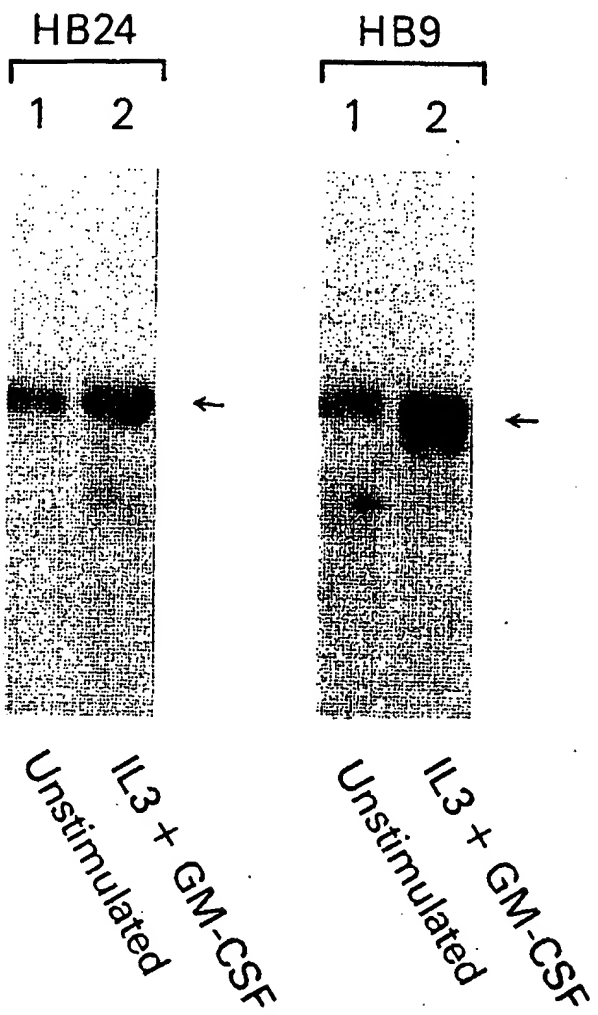

Fig 3. Increased HB24 and HB9 expression following treatment of CD34-positive cells with IL-3 and GM-CSF. RNase protection assays were performed to assess HB24 (left panel) and HB9 (right panel) expression. Lane 1, untreated CD34-positive cells; lane 2, recombinant IL-3– (300 U/mL with a specific activity of 10^8 U/mg protein) and recombinant GM-CSF– (100 U/mL with a specific activity of 5×10^7 CFU/mg protein) treated CD34-positive cells. The cells were treated for 24 hours with cytokines. Ten micrograms of cytoplasmic RNA was analyzed in each lane.

tional regulatory genes[9] (Y. Deguchi, manuscripts submitted for publication), HB9 and HB24, which are selectively present at high levels in CD34-positive cells. Transcripts from both HB9 and HB24 have relatively short half-lives in CD34-positive cells. Treatment of CD34-positive cells with IL-3 and GM-CSF rapidly further increases steady-state mRNA levels for both HB9 and HB24. However, this increase is likely to be transient because unfractionated BM cells depleted of CD34-positive cells express essentially no HB9 or HB24 mRNA, and differentiation of hematopoietic progenitor cells along lineage-specific lines is accompanied by a downregulation of both HB9 and HB24 in CD34-positive cells cultured with IL-3. These results suggest that differentiation of CD34-positive cells along a specific cell lineage likely requires downregulation of both HB9 and HB24.

Previous studies with HB9 and HB24 have shown that both genes are inducible in lymphocytes and present in most lymphoid cell lines examined. In addition, the HB9 gene is present in stromal cells derived from human tonsils. Examination of a limited number of fetal tissues showed HB9 expression in 18 week brain and testis while HB24 was also present in brain, but in addition to developing inferior vena cava. The two genes are likely to be conserved evolutionarily because Southern blots show cross-hybridization with DNA from a variety of other species. In addition, both genes cross-hybridize with mRNAs derived from a murine teratocarcinoma cell line F9[9] (Y. Deguchi, manuscripts submitted for publication). In the present study, the high constitutive levels of expression of HB24 in hematopoietic progenitor cells is relatively unique in that no expression has been found in other adult tissues or primary cells (in the absence of activation) that have been examined. The HB9 gene is expressed constitutively in stromal cells as well as in the CD34-positive BM cells. Unfortunately, these expression studies have yet to provide any insights into the functions of these two genes. Further studies are in progress to attempt to identify the cis DNA sequences that these proteins recognize as well as the other gene products that they regulate. If CD34 and either HB9 or HB24 are coordinately expressed as is suggested by these studies then CD34 gene would be a candidate for regulation by these genes.

Other homeobox genes have been found to be expressed in BM-derived cells as well as in hematopoietic cell lines. For example, HOX 1.1 and HOX 6.1 were isolated from a mouse BM cDNA and found to be expressed in a variety of hematopoietic cell lines.[26] HOX 1.1 was expressed in all cell lines tested including cell lines of erythroid, myeloid, and lymphoid lineage while HOX 6.1 was present in a limited number of the tested cell lines although it was present in at least one cell line from each lineage.[26] The expression of these genes in normal BM cells was not examined. Based on low stringency hybridization studies, it has been estimated that as many as 20 homeobox-containing genes are expressed within the hematopoietic compartment,[26] suggesting that homeodomain-containing proteins are likely to play an important role in the hematopoietic cells. There is also some indirect evidence that homeobox genes may be involved in leukemogenesis. In the mouse myeloid leukemia cell line, WEHI-3B, HOX 2.4 is transcribed constitutively as a result of the insertion of an intracisternal A particle.[27] In addition, 55 of 59 mouse myeloid leukemias tested contained a deletion in the HOX 4.1 locus,[28] and, recently, a translocation found in human acute pre–B-cell lymphoblastic leukemia was found to involve a homeobox gene, PRL, at the chromosomal break point.[29,30] Because the differentiation of hematopoietic progenitor cells along lineage-specific lines likely requires the downregulation of HB24 and HB9, the dysregulation of either HB24 or HB9 expression may impair normal differentiation and contribute to oncogenesis.

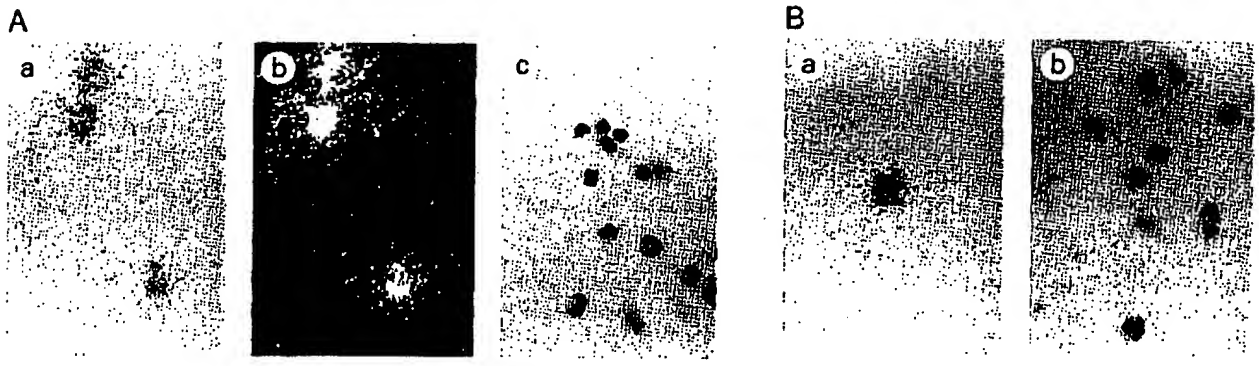

Fig 4. Downregulation of HB24 and HB9 expression following the differentiation of CD34-positive cells cultured in vitro with recombinant IL-3. In situ hybridization assays were performed to assess HB24 (A) and HB9 (B) expression. (A, a) Bright field photomicrograph of CD34-positive cells hybridized with an anti-sense HB24 probe. (A, b) Dark field photomicrograph of the same cells as shown in (A, a). (A, c) Bright field photomicrograph of purified CD34-positive cells that have been cultured with IL-3 for 3 weeks and hybridized with the anti-sense HB24 probe. (B, a) Bright field photomicrograph of CD34-positive cells hybridized with the anti-sense HB9 probe. (B, b) Bright field photomicrograph of purified CD34-positive cells treated with IL-3 for 3 weeks and hybridized with the anti-sense HB9 probe.

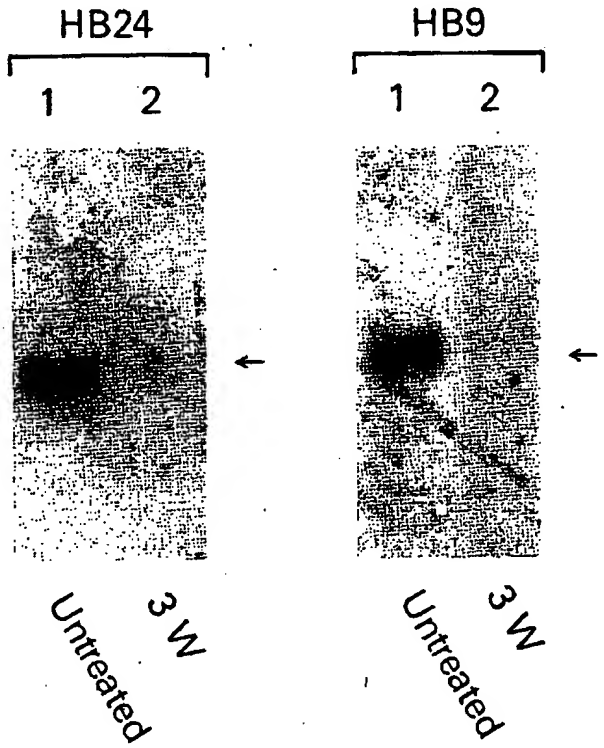

Fig 5. RNase protection assay of HB24 and HB9 expression following the differentiation of CD34-positive cells cultured in vitro with recombinant IL-3. The left panel is from the analysis of HB24 mRNA transcripts and the right panel is from HB9 mRNA transcript. Lane 1, untreated CD34-positive human cells; lane 2, CD34-positive cells treated with recombinant IL-3 (300 U/mL) for 3 weeks. Ten micrograms of total RNA was used in each lane.

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Chapter 3: A. Blood Cell Identification by Staining and Morphology

White blood cells comprise a diverse collection of leukocytes mediating a variety of immunologically related functions. Individual cell types can be microscopically distinguished by gross morphology and by staining with cytochemical dyes. For example, Wright-Giemsa stain, with its combination of acidic and basic dyes, will differentially stain the granules, cytoplasm, and nuclei of various blood cell types as illustrated by some of the images linked to this site (1).

Differential staining morphology of different blood cell types

- red blood cells (**RBCs**):
  cytoplasm = orange-pink to rose
- monocytes:
  cytoplasm = pale gray-blue
  nucleus = deep bluish-purple
- lymphocytes:
  cytoplasm = light blue
  nucleus = deep blue-violet
- neutrophils:
  granules = purple-to-lilac
  cytoplasm = pale pink
  nucleus = deep blue-violet
- eosinophils:
  granules = orange to pink
- basophils:
  granules = deep blue to violet
- platelets:
  central granules = red-purple surrounded by light blue

[Buttons of identical color point to the same web link.]

Morphologically, white blood cells are classified into two broad categories -- granulocytes and mononuclear cells.

Granulocytes (Figure 3-13), which typically have multi-lobed nuclei (often shaped like sausages on a string) and a granular cytoplasm, are comprised of three basic cell types (2):

- neutrophils, also referred to as polymorphonuclear cells, or more simply PMNs
- eosinophils; and
- basophils, the blood-borne precursors of mast cells.

Mononuclear cells, which typically have rounded or kidney-shaped nuclei and often little cytoplasm, are comprised of two basic cell types:

- monocytes (Figure 3-11), the blood-borne precursors of macrophages (Figure 3-12b); and
- lymphocytes, morphologically classified as small and large (Figure 3-10).

  Lymphocytes themselves (Figure 3-9, 2nd ed.) comprise a heterogeneous mixture of functionally distinct cell types, including B lymphocytes, the precursors of antibody-producing plasma cells, several types of T lymphocytes, and natural killer (NK) lymphocytes.

In addition to their distinctive cytochemical staining characteristics, blood cells can be distinguished on a gross level by their average size and granularity as measured by flow cytometry. With a flow cytometer, the optical effects of passing a single cell through a laser light beam can be measured in terms of light scattered by the cell in two directions -- parallel to the beam ("forward scattering" or FSC) and perpendicular to the beam ("side scattering" or SSC). Greater FSC correlates with larger cell size while greater SSC correlates with more granularity in the cytoplasm and nucleus of a cell. A two-dimensional plot of FSC versus SSC for human blood cells, reveals that different cell types exhibit distinct average ranges of size and granularity. Thus, flow cytometry can be used to analyze and even physically isolate different blood cell populations.

With a modified flow cytometer designed to detect fluorescent light stimulated by the laser beam, *i.e.,* a fluorescence-activated cell sorter (FACS), even finer distinctions between different cell populations can be made if they have been treated with fluorescently tagged monoclonal antibodies directed against specific cell surface molecules, generically referred to as cluster of differentiation (CD) antigens.

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  Edited by: L. H. Hoffman, Ph.D.
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- University of Washington Health Sciences Center
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[Chapter 3: Cells & Organs of the Immune System]

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Immunology, January, 1997

Last Updated: June 24, 1997

Result of search for 4169

Welcome, new user.

mononuclear phagocytes

Monocytes and their differentiated products, macrophages. "Mononuclear cells" are leucocytes other than polymorphonuclear cells and include lymphocytes.

Author: anon

There were 1 hits for 4169 in 4169 records.

Result of search for 4885

Welcome, new user.

PBMC

(= peripheral blood mononuclear cells)

A mixture of monocytes and lymphocytes; blood leucocytes from which granulocytes have been separated and removed.

Author: John Lackie

There were 1 hits for 4885 in 4885 records.

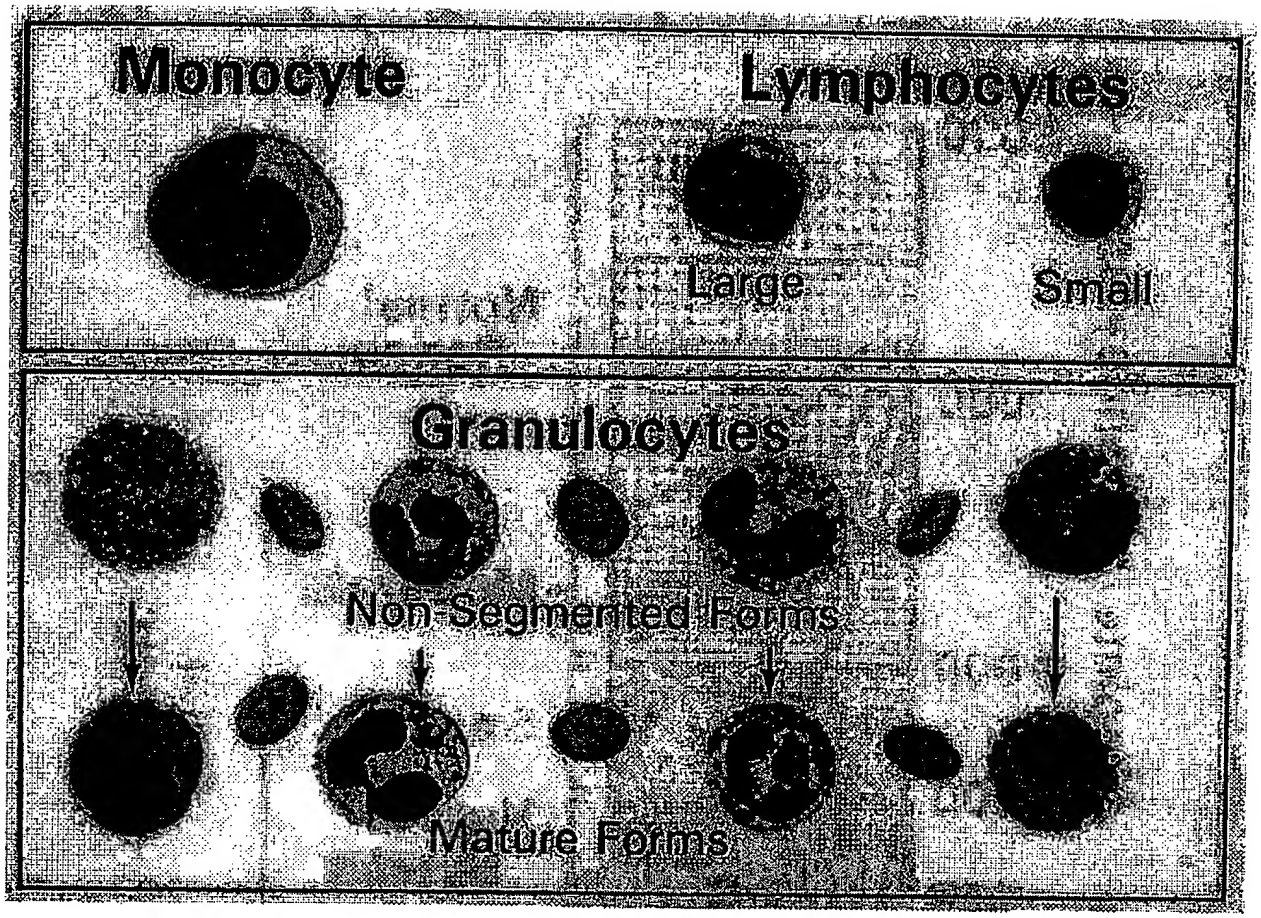

Figure 3-2. Appearance of human mononuclear cells (lymphocytes and monocytes) compared to human granulocytic cells (eosinophils, neutrophils, and basophils) in their nonsegmented and segmented (mature) forms. Erythrocytes are shown for contrast in size.

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